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"Identification of virulent *Capnocytophaga canimorsus* isolates by capsular typing"

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Running title: Capsular typing of *C. canimorsus*

Abstract

Capnocytophaga canimorsus is a dog oral commensal that causes rare but severe infections in humans. *C. canimorsus* was recently shown to be endowed with a capsular polysaccharide implicated in the resistance to the innate immune system of the host. Here we developed the first *C. canimorsus* capsular serotyping scheme. We describe nine different serovars (A to I), which allowed to type 25/25 isolates from human infections but only 18/52 isolates from dog mouths, indicating that the repertoire of capsules in the species is vast. However, three serovars only (A, B, and C) covered 88 % of the human isolates tested (22/25) while they covered only 7.7 % of the dog isolates (4/52). Serovars A, B, and C were found 22.9, 14.6, and 4.2 fold respectively more often among human isolates than among dog isolates, with no geographical bias, implying that isolates endowed with these three capsular types are more virulent for humans than other isolates. Capsular serotyping would thus allow to identify virulent isolates in dogs, which could contribute to the prevention of these infections. To this end, we developed a PCR typing method based on the amplification of specific capsular genes.

Introduction

Capnocytophaga canimorsus are agents of septicemia that often evolve to a septic shock in spite of an adequate treatment (1). Since their discovery in 1961 (2) more than 480 cases of infections were reported in the literature (for a recent review see (3)). With a mortality rate of 30 % and significant morbidity, the prognosis of *C. canimorsus* sepsis is poor (4, 5). Although less frequently reported, meningitis and endocarditis are also associated with *C. canimorsus* infections (3). The genus *Capnocytophaga*, which belongs to the family of *Flavobacteriaceae* in the phylum of *Bacteroidetes* comprises

capnophilic species found in the oral cavities of human and domestic animals. The dogs and cats mouth hosts *C. canimorsus* (6), formerly dysgonic fermenter-2 (DF-2), *C. cynodegmi* (1, 6) and the newly described *C. canis* (7) and "C. stomatis" (8) but only *C. canimorsus* is associated with severe human infections (1, 7, 8). According to studies carried out in different countries, the prevalence of *C. canimorsus* ranges from 19 to 74% in dogs and 21 to 57% in cats (9-13). However, these figures may include *C. canis* and "C. stomatis" that were separated recently from the *C. canimorsus* species. Transmission to humans mostly occurs through dog (97%) or cat (3%) bites, scratches, licks, or simple contact (3, 14). The prevalence of *C. canimorsus* infections was estimated at 0.5 and 0.63 case per million inhabitants per year in Denmark (5) and in the Netherlands (15) respectively but a recent study in the Helsinki area (Finland) estimated the prevalence as high as 4.1 cases per million inhabitants per year (16). *C. canimorsus* infections could thus be under-diagnosed due to the fastidious and slow growth of *C. canimorsus* in culture (1, 17). In addition, the initial clinical manifestations of *C. canimorsus* infections are not specific and their onset can be as late as 8 days after contact with a dog (3, 5). The median age of patients is comprised between 52 and 59 years and a male to female ratio of 3/2 is generally observed (3, 5, 16). Splenectomy and alcohol abuse are common predisposing factors but up to 40% of patients presented no obvious risk factor (18) implying that *C. canimorsus* cannot solely be considered as an opportunistic pathogen.

C. canimorsus strain 5 (Cc5, BCCM/LMG 28512), a strain isolated from a fatal septicemia (19) has a lipooligosaccharide (LOS) and a capsular polysaccharide (CPS) which are genetically and biochemically related (20). The CPS plays a key role in the innate immunity evasion by conferring Cc5 its resistance to phagocytosis by

Journal of Clinical Microbiology

macrophages, to polymyxin B, and to 10 % human serum (20). In addition to being recognized virulence factors for both Gram-negative and -positive bacteria (for review see (21)), CPS are also useful to serotype bacteria and to identify virulent isolates (22, 23). Here we show that 25 isolates of *C. canimorsus* out of 25 from a collection of isolates from human infections are endowed with a CPS and that those polysaccharide structures present a limited variability, with 3 dominant capsular serovars. In addition, a clear enrichment of these dominant capsular serovars was found in human isolates (22/25) as compared to isolates from dog mouths (4/52). Finally, we show that PCR typing can be used to detect these serovars more virulent for humans. This study paves the way to prevention of these dramatic infections.

Material and methods

Bacterial strains, isolates, and culture conditions

Bacterial strains and isolates used in this study are listed in the **Supplementary Tables S1** and **S2**. *C. canimorsus* were grown on heart infusion agar (HIA; BD Difco, Franklin Lakes, NJ, USA) supplemented with 5% sheep blood (SB; Oxoid, Basingstoke, UK) plates (SB plates) for 48h at 37°C with 5% CO_2. *Escherichia coli* were routinely grown in lysogeny broth (LB; Invitrogen, Waltham, MA, USA) at 37°C. Antibiotics used as selective agents were added at the following concentrations: 100 µg/mL ampicillin (AMP) and 50 µg/mL kanamycin (KAN) for *E. coli* and 20 µg/mL gentamicin (GEN), 10 µg/mL erythromycin (ERY), and 10 µg/mL cefoxitin (FOX) for *C. canimorsus*. Unless otherwise stated products were purchased from Sigma-Aldrich (Darmstadt, Germany).

Anti-sera production and adsorption

Bacteria were grown for 2 days on SB plates supplemented with GEN, gently scraped from the agar, resuspended and washed in PBS. Bacteria were fixed overnight in 0.3% paraformaldehyde (PFA), washed in PBS and inoculated to a rabbit to generate polyclonal sera. Sera were generated at the University of Namur (Belgium) or at the Centre d'économie rurale (CER Groupe; Aye, Belgium). The respective Animal Welfare Committees approved the animal handling and procedures. Polyclonal sera were adsorbed by incubation with an excess of PFA-fixed non-capsulated mutant bacteria unless stated otherwise in results. Incubations were done on a rotating wheel at room temperature (RT) and repeated four times. Bacteria were removed by repeated centrifugations. Adsorption efficacy was assessed by immunofluorescence as follow. Glass coverslips were coated with poly-D-lysine (10 µg/mL in PBS, for 1 hour at 37°C), washed and incubated for 30 min at 37°C with 300 µL of a bacterial suspension adjusted to an OD_{600} of 0.25. Coverslips were then washed and bacteria were fixed for 15 min with 4% PFA. Coverslips were washed again and blocked with 1% bovine serum albumin (BSA) for 1 hour at RT. Bacteria were stained with the adsorbed sera (1/1000 in PBS) for 1 hour at RT followed by an incubation with an Alexa Fluor 488-coupled donkey anti-rabbit antibody (1/5000 in PBS; Life technologies, Waltham, MA, USA) or a Texas Red coupled goat anti-rabbit antibody (1/1000 in PBS, Southern Biotech, Birmingham, AL, USA) for 45 min. Coverslips were mounted using mowiol mounting medium and images were acquired with an Axio Imager.Z1 (Zeiss, Oberkochen, Germany) and analyzed using Zen 2012 software (Zeiss).

Mutagenesis by allelic exchange

Journal of Clinical Microbiology

Mutagenesis of Cc6, Cc9, and Cc12 strains was performed as previously described (24). The *C. canimorsus* deletion mutants and the *E. coli* strains used are listed in the **Supplementary Table S2**. Briefly, replacement cassettes with flanking regions spanning approximately 500 base pairs (bp) homologous to regions directly framing targeted genes were constructed with a three-fragment overlapping PCR strategy. First, two PCRs were performed on 100 ng of Cc6, Cc9, or Cc12 genomic DNA with primers 1.1 and 1.2 for the upstream flanking regions and with primers 2.1 and 2.2 for the downstream regions (**Supplementary Table S3**). Primers 1.2 and 2.1 contained an additional 5′ 20-nucleotide extension homologous to the *ermF* insertion cassette. The *ermF* resistance cassettes were amplified from plasmid pMM13 (24) DNA, with primers 3.1 and 3.2. All three PCR products were cleaned and then mixed in equal amounts for PCR using Phusion polymerase (Finnzymes, Espoo, Finland). The initial denaturation was at 98°C for 2 min, followed by 12 cycles without primers to allow annealing and elongation of the overlapping fragments (1 cycle consists of 98°C for 30 s, 50°C for 40 s, and 72°C for 2 min). After the addition of external primers (primers 1.1 and 2.2), the program was continued with 20 cycles (1 cycle consists of 98°C for 30 s, 50°C for 40 s, and 72°C for 2 min 30 s) and finally 10 min at 72°C. Final PCR products consisting of *locus::ermF* insertion cassettes were then digested with *Pst*I and *Spe*I (New England Biolabs, Ipswich, MA, USA) for cloning into the appropriate sites of the *C. canimorsus* suicide vector pMM25 (24). The resulting plasmids were transferred by RP4-mediated conjugative DNA transfer from *E. coli* S17-1 to the corresponding *C. canimorsus* strains to allow integration of the insertion cassette. Transconjugants were then selected for the presence of the *ermF* cassette on erythromycin-containing plates and checked for sensitivity to cefoxitin. Deletion of the appropriate regions was verified by PCR.

Journal of Clinical Microbiology

Western blotting of polysaccharide structures

Bacteria were harvested by gently scraping colonies off the agar surface of GEN SB plate and resuspended in PBS. Bacteria suspensions were adjusted to an OD_{600} of 1 in PBS. 750 µL of the suspension were pelleted and resuspended in 125 µL loading buffer (1% sodium dodecyl sulphate (SDS), 10% glycerol, 50mM dithiothreitol, 0.02% bromophenol blue, 45mM Tris (pH6.8)). Samples were heated for 10 min at 99°C. Proteinase K (VWR Chemicals, Radnor, PA, USA), was added to a final concentration of 50 µg/mL and samples were incubated overnight at 37°C. Subsequently, samples were heated for 10 min at 99°C and proteinase K was added again at the same final concentration. Samples were incubated for 3 hours at 55°C, heated for 5 min at 99°C and loaded on a 12% polyacrylamide gel. After SDS-PAGE (polyacrylamide gel electrophoresis), proteinase K resistant structures were transferred on a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). Membranes were blocked and incubated with polyclonal crude or adsorbed sera (dilutions ranging from 1/400 to 1/8000) followed by incubation with a horseradish peroxidase (HRP)-coupled goat anti-rabbit polyclonal antibody (1/2000; Dako Agilent Technologies, Santa Clara, CA, USA). Membranes were revealed using a chemiluminescent substrate (KLP, Gaithersburg, MD, USA) on an Amersham Imager 600 (GE Healthcare). Blocking and all incubations were conducted in 5% non-fat dry milk diluted in PBS 0.05% Tween.

Capsular serotyping by ELISA

Bacteria suspensions were adjusted to an OD_{600} of 0.5 and were killed by an incubation of 30 min at 70°C. Heat-killed bacteria suspensions were used to coat 96 well plates

Journal of Clinical Microbiology

(ThermoScientific, Waltham, MA, USA) overnight at 4°C. The next day plates were washed to remove unfixed bacteria and blocked for 1 hour at RT with 1% BSA in PBS. Plates were washed and incubated with adsorbed polyclonal serum (1/1000 to 1/5000 in PBS) for 1 hour at RT. Plates were washed again and incubated with HRP-coupled goat anti-rabbit polyclonal antibody for 1 hour at RT (Dako Agilent Technologies; 1/2000 in PBS). Plates were then washed and revealed using 3,3',5,5'-Tetramethylbenzidine (TMB) as a chromogenic substrate.

Capsular serotyping by PCR

Bacteria were grown on SB plates supplemented with GEN and a single colony was resuspended in 100 µL ddH_2O and heated for 15 min at 98 °C. Two microliters were used as template for amplification. PCR detection was performed using the Promega Go Taq® G2 polymerase (Madison, WI, USA) under the following conditions: an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 45 s, extension at 72°C for 1 min and 30 s, and a final extension at 72°C for 7 min.

Synteny analysis

Synteny statistics were obtained using the MicroScope PkGDB synteny statistics tool (https://www.genoscope.cns.fr/agc/microscope/home/index.php) (25). Putative orthologous relations based on the bi-directional best hit (BBH) criterion were considered for at least 35% of sequence identity on 80% of the length of the smallest protein. For the synteny analysis, all possible kinds of chromosomal rearrangements are allowed (inversion, insertion/deletion) and the gap parameter, representing the

maximum number of consecutive genes which are not involved in a synteny group, is set to five genes.

Statistical analysis

Statistical significance was evaluated by Fisher's exact tests using the BiostaTGV website (https://marne.u707.jussieu.fr/biostatgv).

Accession numbers

Accession numbers of genes used in this study are listed in **Supplementary Table S4**.

Results

Capsular serotyping identifies 5 serovars in a collection of *C. canimorsus* isolated from human infections

The prevalence of the capsular serovar of strain Cc5 was tested in a collection of 25 *C. canimorsus* isolated from human infections (**Supplementary Table S1**). Whole bacteria were digested with proteinase K and bacterial polysaccharides were analyzed by western blot using an anti-serum directed against Cc5 bacteria and adsorbed with the non-capsulated Cc5 transposon mutant Y1C12 (20, 26). The serum recognized a high molecular weight (MW) band (>250 kDa) in the extracts from Cc5 and from ten other isolates, namely Cc1 (BCCM/LMG 11511; CCUG 17234; strain P810; strain SSI P810), Cc2, Cc3, Cc10 (BCCM/LMG 11541, CCUG 24741, ATCC 35978, CDC C8936), Cc13, Cc15, Cc21 (CCUG 60839), Cc22 (CCUG 20318), Cc24 (CCUG 67384), and Cc25 (CCUG 66222) (**Figure 1A**). Since this band was identified as the CPS of Cc5 (20), we concluded that the capsular serovar of Cc5 was shared with these 10 isolates

representing 44% of our collection of human isolates. We named this capsular serovar A.

To determine the capsular serovar of the 14 non-A human isolates, 9 new anti-sera were raised and tested by western blot on polysaccharide extracts from these 14 isolates. The antisera raised against Cc6, Cc9 (BCCM/LMG 11510, CCUG 12569, CDC A3626), Cc12 (type strain, ATCC 35979, CDC 7120, CCUG 53895), and Cc4 allowed detecting a high MW polysaccharide, most likely corresponding to a CPS (**Figure 1**) in all the 14 isolates. The anti-Cc6 serum recognized a high MW polysaccharide structure in Cc6 but also in Cc8, Cc11, Cc16, Cc17, Cc18, and Cc23 (CCUG 48899) (**Figure 1B**). This serovar, named B, had thus a prevalence of 28% in our collection of human isolates, with 7 isolates positive out of 25. The anti-Cc9 serum recognized a high MW polysaccharide structure in Cc9, Cc14, Cc19, and Cc20 (CCUG 55909) (**Figure 1C**). This serovar, named C had thus a prevalence of 16%. The anti-Cc12 serum recognized a high MW polysaccharide structure in Cc12 and Cc7 (**Figure 1D**). The prevalence of this serovar, named D, was of 8%, thus more limited than that of serovars A, B, and C. Finally, the anti-Cc4 serum recognized a high MW polysaccharide band only in Cc4. This serovar had thus a prevalence of only 4% and was named E (**Figure 1E**).

In order to confirm that the high MW bands recognized are CPS, we next attempted to generate non-capsulated deletion mutants of Cc6, Cc9, Cc12, and Cc4 (**Supplementary Table S2**). Since the capsule of Cc5 is made of the same sugars as the LOS O-chain, we decided to generate rough non-capsulated mutants. To this aim we sequenced the genomes of the isolates Cc6, Cc9, and Cc4 and used the previously published genome of Cc5 (GenBank: CP002113) (27) and draft genome of Cc12 (GenBank: CDOE00000000.1) (28). Homologs of Cc5 *wbuB* gene (*Ccan_23370*), which is the gene

Journal of Clinical Microbiology

mutated in the LOS/CPS mutant Y1C12 and encodes a N-acetyl-fucosamine (FucNAc) transferase (20), were found in the genomes of Cc6 (*Cc6_1430029*) and Cc9 (*CCAN9_740038*) but not in those of Cc12 and Cc4. In the latter genomes we identified homologs of Cc5 *wbtA* (*Ccan_23400*) that is mutated in the LOS/CPS mutant Y1D1 (20) of Cc5 and encodes an UDP-N-acetylglucosamine 4,6-dehydratase (*CCAN12_760057*, and *CC4_530070* respectively) (20). The *wbuB* genes were thus mutated in Cc6 and Cc9 while gene *wbtA* was mutated in Cc12. The polysaccharide extracts from the mutants of Cc6, Cc9, and Cc12, analyzed by western blot with the anti-Cc6, anti-Cc9, and anti-Cc12 sera, did not contain the high MW band indicating that it was indeed a CPS (**Figure 1B, C** and **D**). Gene *wbtA* from Cc4 could not be mutated despite several attempts and hence we could not formally prove that the high MW polysaccharide is a CPS related to the LOS. Nevertheless, the presence of a *wza* homolog, encoding for the capsular transporter across the outer membrane, suggests that Cc4 is indeed endowed with a capsule.

We thus conclude that the 25 *C. canimorsus* human isolates of our collection are all endowed with a CPS and that the antigenic repertoire of these CPS is limited since 88% of the isolates (22/25) belong to serovars A, B, and C. Interestingly, the distribution of serovars A, B, and C is not affected by a geographical bias since each serovar was found in isolates from at least three different countries (**Supplementary Table S5**).

Prevalence of capsular serovars A, B, and C among *C. canimorsus* isolated from dog mouths

We next assessed the prevalence of the capsular serovars A to E among a collection of 52 isolates of *C. canimorsus* from dog mouths (**Supplementary Table S1**) (7). To this

Journal of Clinical Microbiology

aim we set up an ELISA screening using entire heat-killed bacteria. Since we needed sera that were specifically recognizing the CPS, for serovar A, we used the Y1C12-adsorbed anti-Cc5 serum; for serovars B, C, and D, we adsorbed the crude anti-Cc6, anti-Cc9, and anti-Cc12 sera with Cc6 *wbuB,* Cc9 *wbuB*, and Cc12 *wbtA* mutant bacteria respectively. Due to the lack of a non-capsulated Cc4 mutant strain, we adsorbed the anti-Cc4 serum (serovar E) with the 24 other human isolates belonging to different capsular serovars (**Figure 1E**). The efficacy of the different adsorptions was validated by immunofluorescence staining and microscopy analysis (**Supplementary Figure S1**). The five adsorbed sera were then used to test our collection of dog isolates by ELISA. The reactivity of each isolate was calculated with respect to that of the type strain of each serovar (Cc5 for A, Cc6 for B, Cc9 for C, Cc12 for D, and Cc4 for E). The non-capsulated mutant strains were used as negative controls. The results of the screening are summarized in **Table 1**. Only two isolates, CcD68 and CcD105, were positive for serovar A with a reactivity of 43% ± 7 and 107% ± 28 respectively. The high MW polysaccharidic structures of these isolates were analyzed by western blot and only the strongly reacting CcD105 displayed a serovar A capsule (**Supplementary Figure S2A**). For serovar B, only isolate CcD68 was found to be positive (110% ± 11) by ELISA and by western-blot (**Supplementary Figure S2B**). For serovar C, isolates CcD43 and CcD130 were positive by ELISA (86% ± 5 and 108% ± 26 reactivity respectively) and western blot (**Supplementary Figure S2C**). For serovar D, three isolates were strongly recognized by ELISA and confirmed by western blot: CcD16 (86% ± 14), CcD89 (95% ± 9), and CcD117 (99% ± 12) (**Supplementary Figure S2D**). Finally for serovar E, isolate CcD96 displayed a high reactivity of 118% ± 37 and isolates CcD20 and CcD106 displayed intermediate reactivities of respectively 57% ± 24 and 59% ± 24 while some

Journal of Clinical Microbiology

other isolates presented a limited reactivity. All the isolates with a value equal or higher than 30 % were checked by western blot and only one isolate, CcD96, was confirmed to belong to serovar E (**Supplementary Figure S2E**). The results from the ELISA and the western blot analyses are summarized in **Figure 2**. While all the human isolates belonged to serovars A, B, C, D, or E, 84.6% of the dog isolates were left non-typeable. In conclusion, the prevalence of serovar A was 22.9 fold higher in human isolates than in dog isolates (Fisher's exact test, $p=6.45.10^{-6}$) while the prevalence of serovar B was 14.6 fold higher (Fisher's exact test, p=0.00123). A 4.2 fold increase was found for the serovar C, but it was not statistically significant (Fisher's exact test, p=0.0831). Finally, there was no significant difference in the prevalence of serovars D and E (p values of 0.657 and 0.547 respectively in Fisher's exact test).

There is a high capsular variability among the isolates from dog mouths

To investigate the variability of the capsular serovars in the 44 untyped dog isolates, we generated sera against 4 isolates randomly chosen (CcD37, CcD63, CcD101, and CcD129). Since we could not generate uncapsulated mutants because the genomes of these isolates are not available, the anti-sera were adsorbed using a mix of the 25 *C. canimorsus* human isolates. After validating the adsorption efficacy by immunofluorescence (**Supplementary Figure S1**) we screened the 52 dog isolates by ELISA (**Table 1**). The adsorbed anti-CcD37 serum reacted not only with CcD37 but also with CcD13, CcD52, CcD113, CcD118, and CcD124 with reactivities comprised between 83 and 111%. All these reactions were confirmed by western blot (**Supplementary Figure S2F**). This serovar, named F, had thus a prevalence of 11.5% among dog isolates (6/52). The adsorbed anti-CcD63 reacted with the CPS of CcD63 but with no

other isolate (**Supplementary Figure S2G**). This serovar, G, had thus a reduced prevalence of 1.9% (1/52). The adsorbed anti-CcD101 serum reacted with only one other isolate, (CcD53) but this isolate did not show any CPS (**Supplementary Figure S2H**). This serovar, H, had thus a prevalence of 1.9 % (1/52). Finally the adsorbed anti-CcD129 serum reacted by ELISA and western blot with the CPS of CcD129 and CcD33 (**Supplementary Figure S2I**). This serovar, I, had thus a prevalence of 3.8% (2/52). There were no significant differences in the prevalence of serovars F, G, H, and I between dog and human isolates (p values of 0.169 for F and 1 for G, H, and I in Fisher's exact test), but while five serovars covered the 25 human isolates (100 %), nine serovars covered only 18 dog isolates (34.6 %) (**Figure 2**). This result indicates there is a higher variability of capsular serovars among dog isolates than among human isolates.

Detection of the capsular serovars A to E by PCR

Our data so far clearly show that the capsular serotyping could help identifying dogs hosting *C. canimorsus* isolates that are more virulent for humans than others. Since immunological screening methods are somehow difficult to implement in diagnostics laboratories, we tried to develop a PCR-based method using different oligonucleotides couples that would allow the identification of the 5 serovars found among human isolates.

We thus first compared the capsule and LOS biosynthesis loci in the seven available genomes of *C. canimorsus* isolates belonging to the five serovars (Cc5, Cc2, Cc6, Cc11, Cc9, Cc12, and Cc4) (**Figure 3**). Looking for a gene that was specific to serovar A isolates (Cc5, Cc2), we identified an A4GalT-like glycosyltransferase gene (*Ccan_23210* and *CCAN2_1920004* in Cc5 and Cc2 respectively) (20). Two amplimers were designed

and our complete *C. canimorsus* collection was tested by PCR. As shown in **Figure 4** and **Table 2,** this analysis detected all serovar A isolates (11 human- and one dog-isolates) and no other isolate.

Regarding serovar B, we could not identify any gene that was unique to the Cc6 and Cc11 genomes (**Figure 3**). However, while genes *CC6_1430035* and *CCAN11_10027*, both encoding a putative family 1 glycosyltransferase, were exactly conserved in Cc5 (serovar A), they were not in Cc2 (also serovar A). Aligning *CC6_1430035* and *CCAN11_10027* with their homologs from Cc2 (*CCAN2_1430008*) and Cc9 (serovar C) (*CCAN9_740032*) (20) revealed a difference in the 16 base pairs immediately downstream of the start codon (**Supplementary Figure S3**). Since both serovar B isolates (Cc6 and Cc11) had the exact same gene sequence, shared by only one of the two serovar A isolates (Cc5), we tested whether the exact same gene sequence would not be shared by all serovar B isolates. We thus designed two oligonucleotides to amplify this specific gene region and, as shown in **Figure 4** and **Table 2**, by this PCR, we could indeed detect all the 7 serovar B human isolates as well as the only serovar B dog isolate (CcD68). As expected we could also detect Cc5 but two other serovar A isolates, namely Cc15 and Cc24, as well. Surprisingly, the PCR gave a positive result for one dog isolate (CcD57) that did not belong to any of the 5 serovars (**Table 1**, **Supplementary Figure S2E** and **Supplementary Figure S4A**) and thus represents a false positive. Nevertheless, with this PCR we could detect all serovar B isolates of our collection and this analysis, if combined with the one specific for the serovar A, allowed the discrimination between serovars A and B. Indeed, serovar B isolates are positive for PCR B but negative for PCR A.

Regarding serovar C, we could not identify any gene unique to the Cc9 genome but *CCAN9_740031*, encoding a putative O antigen polymerase (*wzy*) had an homolog only in one serovar A isolate, namely Cc2 (**Figure 3**). We thus tested by PCR whether this gene would be shared by all serovar C isolates. As shown in **Figure 4** and **Table 2**, we could detect all serovar C isolates namely the 4 from humans (Cc9, Cc14, Cc19) and Cc20) as well as the two from dogs (CcD43 and CcD130). This PCR thus allows the detection of the serovar C isolates and, if combined with the PCR for the serovar A, to discriminate between these two serovars. Indeed, serovar C isolates are positive for PCR C but negative for PCR A.

Concerning serovar D, the Cc12 LOS/CPS locus was previously shown to be very divergent from the ones of serovars A, B, and C isolates with a limited number of conserved genes (20) (**Figure 3**). We chose to amplify gene *CCAN12_760043* encoding a putative lipopolysaccharide biosynthesis O-acetyl transferase (WbbJ) that had no homologs in all the other serovars loci. As shown in **Figure 4** and **Table 2**, this PCR exclusively detected the serovar D isolates and it detected them all (Cc12, Cc7, CcD16, CcD89, and CcD117).

Finally, as for Cc12, the serovar E strain Cc4 LPS/CPS locus strongly diverged from the ones of all the other serovars (**Figure 3**). We thus chose as target gene a Cc4 unique gene, namely *CC4_530066*, encoding a glycosyltransferase 1 family protein. As shown in **Figure 4** and **Table 2**, this PCR detected the Cc4 and CcD96 serovar E isolates. Among the other isolates, only CcD10 gave a positive result although it did not react with the E antiserum (**Table 1** and **Supplementary Figure S4B**) and could thus be considered as a false positive. In summary, in order to determine the serovar of a *C. canimorsus* isolate, the five (A, B, C, D, and E) PCR should be performed and the

Journal of Clinical Microbiology

results interpreted as follows: i) all isolates that are positive for PCR A belong to serovar A; ii) isolates that are positive for PCR B belong to serovar B if they are not positive for PCR A; iii) isolates that are positive for PCR C are serovar C if they are not positive for PCR A; iv) isolates that are positive for PCR D are serovar D; v) isolates that are positive for PCR E are serovar E (**Table 2** and **Table 3**)

In conclusion, capsular serotyping can be done by PCR (**Table 2** and **Table 3**) with a very limited margin of error (2 false positive dog isolates).

Next, given the higher prevalence of serovars A, B, and C (22/25) among human isolates, we decided to develop a PCR that would allow to detect all serovar A, B, and C isolates. To this aim, taking advantage of the high similarity among the LOS/CPS loci of the isolates belonging to serovars A, B, and C, we designed two amplimers specific to the conserved region of the putative glycosyltransferase *wfdR* orthologs genes of serovar A (*Ccan_23240* in Cc5 and *CCAN2_1430002* in Cc2), serovar B (*CC6_1430040* in Cc6 and *CCAN11_2010013* in Cc11), and serovar C (*CCAN9_740027*). As shown in **Figure 4** and **Table 2**, by this PCR we could detect all the isolates belonging to serovars A, B, and C. Among the non-A, -B, or -C isolates, only CcD77 gave an amplification but not of the same size (**Figure 4**). This PCR, allowing to identify fast and specifically all the *C. canimorsus* isolates belonging to serovars A, B, or C (**Table 3**) could thus be a valuable tool in terms of prevention.

Discussion

Here we show that all 25 out of 25 *C. canimorsus* isolated from human infections and 18 dog isolates out of 18 tested are endowed with a CPS. We thus confirm our previous observation where a capsular-like polysaccharide structure was found in ten human

Journal of Clinical Microbiology

isolates (20). This result further reinforces the commonality of the presence of a CPS in *C. canimorsus*. In addition, we developed a serotyping scheme based on the capsular antigens and we described nine serovars (A to I). The LOS and CPS synthesis are genetically linked in strain Cc5, resulting in similar polysaccharide units compositions in both structures (20). For serovars B to I, we also found shared epitopes between the CPS and LOS (data not shown). Even more, the antiserum directed against the CPS/LOS from serovar C recognized the LOS but not the CPS from some serovar A isolates (data not shown), revealing some complexity in the CPS/LOS relation. Because of this complexity and because it is the CPS rather than the LOS that impacts the host-pathogen interaction (20), we based our typing scheme on the CPS only. However, because of this cross-reaction, the distinction between serovars A and C must be done by western blotting and not by immuno-fluorescence or ELISA. Because western blotting is a tedious technique for clinical laboratories, we set up a PCR method for the capsular serotyping. The cross-reaction between the LOS of serovar A and some strains of serovar C also appeared when the typing was done by PCR but combining the two PCR reactions allows to determine the serovar without any ambiguity. Further work will be required to understand the molecular mechanisms underlying these LOS cross-reactions but carbohydrate chemistry always represents a long-term project.

The nine serovars described covered only 18 dog isolates out of 52 tested while five serovars only covered the 25 human isolates. Thus, there was a high variety of capsular serovars among dog isolates. In contrast, only three serovars (A, B, and C) covered 88 % of the human isolates tested (22/25) while they covered only 4 dog isolates (7.7 %). There was thus a very strong enrichment of serovars A, B, and, to a lesser extend C in human isolates as compared to dog ones. Interestingly, these three dominant capsular

Journal of Clinical Microbiology

serovars were not restricted to a geographical area but were rather distributed worldwide. This observation clearly indicates that the strains belonging to serovars A, B, and, possibly C, are more virulent for humans than strains from the other serovars. This sets the bases for the prevention of these severe infections. To this aim, one could envision the detection of potentially more dangerous dogs using a PCR reaction carried out directly on the dog's saliva and monitoring simultaneously the three more virulent serovars. Our results on collection isolates have indeed shown that PCR is reliable with a very limited number of false positives and, in our experience, no false negative. Owners of a dog hosting a serovar A, B, or C strain should be educated to limit the contact with the dog's saliva and if a bite or a lick occurs, to apply strict hygiene measures. In addition, splenectomized and more generally immunocompromized persons should not consider adopting a dog hosting a virulent *C. canimorsus* strain.

Ideally, more human isolates should be serotyped to reinforce the correlation between some capsular serovars and human infections but their collection is very tedious due to the rarity of the disease and the fastidious character of these bacteria.

There was no significant difference in the distribution of capsular serovars D and E among dog (4/52) and human isolates (3/25), which suggests that they are probably not more virulent than most dog strains. This observation leads to the conclusion that, while a majority of the patients (88 % of our sample) are infected with virulent strains (A, B, possibly C), a minority of patients (12 % of our sample) could have been infected by strains that belong to a less virulent serovar (D and E). This is consistent with the fact that some patients were obviously at risk while others had no history of immune deficiency. In agreement with this hypothesis, the patient infected with Cc4 (serovar E) was highly immunocompromised (29) and the patient infected with Cc12 (serovar D)

Journal of Clinical Microbiology

was splenectomized (2) (**Supplementary Table S1**). Hence, splenectomized and more generally immunocompromised persons should be extremely cautious when interacting with dog hosting *C. canimorsus* regardless of the serovar of the latter.

It is likely that it is the capsule itself that confers an enhanced virulence to serovars A, B, and C, as is classical for other pathogens (21). In support of this, the capsule of the type strain Cc5 has been recently shown to provide resistance to phagocytosis by macrophages, to killing by 10% human serum and to killing by the cationic antimicrobial peptide polymyxin B (20). These results suggest that the serovar A CPS could indeed participate to the innate immune evasion in humans. Ideally, these *in vitro* data should be reinforced by *in vivo* studies but the lack of a relevant sepsis animal model to study *C. canimorsus* infections prevents such confirmation. Further *in vitro* work could determine if the capsular serovars A, B, and C provide the strains a higher resistance to the innate immune system. However, we cannot exclude that other virulence factors could be genetically linked to some capsular serovars. It would thus be interesting to compare the whole genomes looking for genes that would be shared in serovars A, B, and C strains and absent in serovars F, G, H, and I strains.

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Journal of Clinical Microbiology

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Figure legends

563 **Figure 1. Capsular serotyping of *C. canimorsus* isolates from human infections.**
564 Western blot analysis of proteinase-K treated lysates of *C. canimorsus* human isolates
565 using the following sera: Y1C12 adsorbed anti-Cc5 (A), anti-Cc6 (B), anti-Cc9 (C), anti-
566 Cc12 (D), and anti-Cc4 (E). Non-capsulated mutants Cc5 Y1C12, Cc6 Δ*wbuB*, Cc9
567 Δ*wbuB*, and Cc12 Δ*wbtA* were used as controls in panels A, B, C, and D respectively.
568 Numbers correspond to molecular mass markers in kDa.
569
570 **Figure 2. Prevalence of capsular serovars A to I in *C. canimorsus* isolated from**
571 **human infections and dog mouths.** Summary of capsular serovars A to I prevalence
572 in human (A) or dogs (B) isolates.
573
574 **Figure 3. Synteny analysis of LOS/CPS loci in the A, B, C, D, E capsular serovars.**
575 Comparison of the LOS/CPS-biosynthesis and transport genetic loci of the seven *C.*
576 *canimorsus* isolates whose genomes were sequenced. The boxes indicate different
577 genomic loci. Homologs of the Cc5 genes are indicated in grey. The genes amplified by
578 the A, B, C, D, and E serovar specific PCR are indicated in green, blue, orange, black,
579 and yellow respectively. The target genes amplified by the ABC serovars specific PCR
580 are in red. Genes indicated in white are isolate specific genes likely involved in
581 LOS/CPS biosynthesis. The hatched pattern indicates genes likely unrelated to
582 LOS/CPS biosynthesis and transport. Fragmented genes are marked with (f). Note that
583 the genomes of Cc2, Cc4, Cc6, Cc9, Cc11, and Cc12 are draft genomes. For the sake
584 of simplicity genes are not represented to scale.
585

Journal of Clinical Microbiology

586 **Figure 4. Capsular typing by PCR.** PCR detection of capsular serovars A, B, C, D, and
587 E in *C. canimorsus* human and dog isolates using the oligonucleotides given in **Table**
588 **S3**. *C. canis* (type strain CcD38, LMG 29146, DSM 101831) and *C. cynodegmi* (type
589 strain Ccyn ATCC 49044) were used as negative controls.
590

Journal of Clinical Microbiology

591 **Table 1: Capsular serotyping of *C. canimorsus* dog isolates by ELISA**
592

Strain/ Isolate	Capsular serovar								
	A	B	C	D	E	F	G	H	I
Cc5	100 ± 0	20 ± 8	27 ± 11	24 ± 6	17 ± 2	13 ± 4	14 ± 5	10 ± 4	13 ± 5
Cc5 Y1C12	14 ± 6	nd	nd	nd	nd	nd	nd	nd	nd
Cc6	32 ± 7	100 ± 0	24 ± 12	20 ± 4	13 ± 2	14 ± 6	12 ± 3	11 ± 4	14 ± 7
Cc6 Δ*wbuB*	nd	14 ± 7	nd	nd	nd	nd	nd	nd	nd
Cc9	15 ± 3	17 ± 7	100 ± 0	22 ± 4	17 ± 5	14 ± 5	13 ± 4	10 ± 3	14 ± 4
Cc9 Δ*wbuB*	nd	nd	20 ± 7	nd	nd	nd	nd	nd	nd
Cc12	19 ± 7	15 ± 5	23 ± 8	100 ± 0	18 ± 0	14 ± 5	15 ± 5	10 ± 3	13 ± 5
Cc12 Δ*wbtA*	nd	nd	nd	20 ± 5	nd	nd	nd	nd	nd
Cc4	16 ± 3	14 ± 3	30 ± 10	26 ± 5	100 ± 0	13 ± 5	13 ± 4	10 ± 2	13 ± 5
CcD3	18 ± 7	11 ± 4	18 ± 5	19 ± 3	14 ± 6	10 ± 3	12 ± 1	15 ± 4	12 ± 6
CcD5	17 ± 4	13 ± 8	16 ± 4	22 ± 4	13 ± 6	11 ± 5	13 ± 2	14 ± 6	14 ± 6
CcD6	18 ± 10	11 ± 5	20 ± 7	18 ± 3	15 ± 7	11 ± 3	14 ± 2	17 ± 4	16 ± 10
CcD10	17 ± 8	12 ± 5	18 ± 3	17 ± 2	17 ± 5	10 ± 1	14 ± 2	15 ± 6	14 ± 6
CcD13	16 ± 7	11 ± 5	21 ± 4	17 ± 2	12 ± 5	99 ± 1	12 ± 1	13 ± 4	12 ± 5
CcD16	18 ± 7	10 ± 4	17 ± 4	86 ± 14	11 ± 5	10 ± 3	13 ± 1	15 ± 5	12 ± 5
CcD18	19 ± 9	12 ± 6	17 ± 5	15 ± 2	28 ± 12	10 ± 2	15 ± 1	17 ± 6	13 ± 6
CcD20	17 ± 6	11 ± 6	19 ± 3	17 ± 3	57 ± 24	11 ± 3	12 ± 1	14 ± 4	12 ± 5
CcD25	15 ± 6	11 ± 5	18 ± 4	17 ± 2	12 ± 5	9 ± 2	11 ± 1	14 ± 4	12 ± 3
CcD33	20 ± 9	11 ± 6	17 ± 6	22 ± 2	13 ± 5	10 ± 3	12 ± 2	13 ± 4	106 ± 30
CcD34	16 ± 9	10 ± 4	16 ± 3	14 ± 2	13 ± 6	10 ± 2	12 ± 1	13 ± 4	13 ± 6
CcD35	14 ± 7	12 ± 4	15 ± 5	12 ± 1	12 ± 4	11± 3	12 ± 2	14 ± 3	12 ± 5
CcD37	15 ± 4	9 ± 3	19 ± 2	16 ± 0	12 ± 4	100 ± 0	11 ± 1	12 ± 4	11 ± 5
CcD39	14 ± 4	10 ± 5	18 ± 3	22 ± 2	14 ± 6	9 ± 2	11 ± 1	12 ± 3	12 ± 5
CcD40	16 ± 8	11 ± 5	19 ± 4	19 ± 4	12 ± 5	10 ± 2	13 ± 2	16 ± 5	13 ± 6
CcD43	20 ± 10	24 ± 14	86 ± 5	17 ± 1	13 ± 5	10 ± 3	12 ± 1	16 ± 1	14 ± 2
CcD44	15 ± 8	9 ± 4	25 ± 7	16 ± 0	12 ± 5	8 ± 1	11 ± 1	12 ± 4	11 ± 5
CcD47	16 ± 6	11 ± 6	17 ± 3	18 ± 0	12 ± 5	8 ± 2	12 ± 1	14 ± 3	11 ± 4
CcD51	15 ± 7	14 ± 5	18 ± 4	20 ± 4	11 ± 5	9 ± 2	12 ± 0	14 ± 4	12 ± 5
CcD52	16 ± 8	11 ± 7	16 ± 5	20 ± 6	11 ± 5	83 ± 4	13 ± 2	14 ± 4	12 ± 5
CcD53	19 ± 8	12 ± 6	17 ± 2	18 ± 2	12 ± 5	9 ± 2	14 ± 2	41 ± 7	11 ± 4
CcD57	17 ± 6	28 ± 18	21 ± 4	23 ± 12	32 ± 10	9 ± 2	13 ± 1	13 ± 4	12 ± 5
CcD58	18 ± 7	11 ± 5	17 ± 3	22 ± 3	34 ± 11	11 ± 2	14 ± 1	15 ± 5	13 ± 5
CcD63	15 ± 9	11 ± 5	17 ± 1	17 ± 0	29 ± 11	11 ± 5	100 ± 0	14 ± 5	12 ± 5
CcD68	43 ± 7	110 ± 11	16 ± 5	18 ± 0	13 ± 5	9 ± 2	13 ± 1	14 ± 5	11 ± 5
CcD69	14 ± 7	11 ± 6	16 ± 3	13 ± 2	12 ± 5	8 ± 3	12 ± 1	12 ± 4	11 ± 5
CcD71	15 ± 6	11 ± 5	17 ± 6	16 ± 2	13 ± 6	10 ± 2	13 ± 1	14 ± 5	13 ± 5
CcD73	19 ± 7	12 ± 7	17 ± 0	23 ± 3	15 ± 6	15 ± 7	13 ± 5	18 ± 7	13 ± 5
CcD76	13 ± 6	16 ± 8	14 ± 1	15 ± 3	15 ± 7	15 ± 7	13 ± 4	12 ± 4	13 ± 4
CcD77	14 ± 9	13 ± 4	17 ± 7	14 ± 2	15 ± 7	14 ± 6	12 ± 5	10 ± 2	13 ± 4
CcD80	16 ± 11	13 ± 4	19 ± 5	17 ± 3	30 ± 9	13 ± 5	13 ± 5	10 ± 4	13 ± 5
CcD81	16 ± 5	12 ± 4	23 ± 7	19 ± 4	22 ± 5	12 ± 5	12 ± 5	9 ± 3	11 ± 4
CcD84	17 ± 8	14 ± 7	13 ± 1	18 ± 2	29 ± 16	12 ± 5	12 ± 3	14 ± 4	13 ± 4
CcD89	17 ± 6	15 ± 1	20 ± 6	95 ± 9	14 ± 9	14 ± 5	13 ± 4	13 ± 5	13 ± 5

CcD96	20 ± 8	14 ± 1	19 ± 7	15 ± 1	118 ± 37	12 ± 4	11 ± 4	9 ± 3	13 ± 5
CcD101	18 ± 5	12 ± 3	13 ± 4	16 ± 1	13 ± 7	12 ± 5	10 ± 3	100 ± 0	12 ± 4
CcD104	16 ± 9	14 ± 1	18 ± 7	21 ± 4	31 ± 11	11 ± 4	10 ± 3	9 ± 3	11 ± 4
CcD105	107 ± 28	14 ± 2	16 ± 8	17 ± 5	14 ± 8	13 ± 5	11 ± 4	9 ± 3	12 ± 4
CcD106	13 ± 8	16 ± 1	21 ± 11	17 ± 2	59 ± 24	13 ± 5	11 ± 4	9 ± 3	13 ± 5
CcD113	15 ± 11	16 ± 2	19 ± 9	14 ± 0	14 ± 6	108 ± 6	11 ± 4	9 ± 4	12 ± 4
CcD115	15 ± 9	16 ± 2	19 ± 9	21 ± 4	16 ± 8	12 ± 4	10 ± 4	9 ± 3	12 ± 5
CcD116	14 ± 8	14 ± 2	19 ± 5	18 ± 2	15 ± 7	12 ± 5	12 ± 5	9 ± 3	13 ± 5
CcD117	19 ± 10	16 ± 1	19 ± 8	99 ± 12	14 ± 7	13 ± 6	13 ± 5	9 ± 3	13 ± 5
CcD118	15 ± 7	14 ± 1	20 ± 9	19 ± 4	15 ± 7	111 ± 5	12 ± 4	10 ± 3	12 ± 5
CcD119	15 ± 8	16 ± 2	16 ± 9	28 ± 22	15 ± 9	12 ± 4	11± 4	12 ± 5	12 ± 4
CcD120	15 ± 8	17 ± 3	17 ± 5	23 ±7	16 ± 8	13 ± 5	12 ± 4	10 ± 3	12 ± 4
CcD122	12 ± 8	14 ± 1	17 ± 6	13 ± 1	14 ± 8	13 ± 5	12 ± 4	9 ± 2	12 ± 4
CcD124	15 ± 8	16 ± 1	19 ± 8	19 ± 4	15 ± 7	109 ± 9	11 ± 4	9 ± 3	12 ± 4
CcD126	13 ± 7	14 ± 1	16 ± 6	21 ± 3	15 ± 7	13 ± 6	11 ± 4	11 ± 4	12 ± 4
CcD129	13 ± 9	15 ± 2	21 ± 6	20 ± 2	16 ± 8	12 ± 5	14 ± 6	10 ± 2	100 ± 0
CcD130	13 ± 7	29 ± 11	108 ± 26	19 ± 2	11 ± 6	15 ± 6	12 ± 4	10 ± 4	13 ± 5
CcD131	12 ± 7	14 ± 2	17 ± 6	19 ± 3	14 ± 6	13 ± 6	11 ± 4	10 ± 3	12 ± 4

Capsular serotyping was determined by ELISA on entire heat-killed bacteria. The following sera were used: Y1C12 adsorbed anti-Cc5 (A), Cc6 Δ*wbuB* adsorbed anti-Cc6 (B), Cc9 Δ*wbuB* adsorbed anti-Cc9 (C), Cc12 Δ*wbtA* adsorbed anti-Cc12 (D), anti-Cc4 adsorbed with all human isolates except Cc4 (E), anti CcD37 adsorbed with all human isolates (F), anti CcD63 adsorbed with all human isolates (G), anti CcD101 adsorbed with all human isolates (H), and anti CcD129 adsorbed with all human isolates (I). The readout of the ELISA was absorbance but results are expressed here as percentage of reactivity calculated with respect to the absorbance value obtained for the capsular type strain. Values are the mean (± standard deviation, SD) of at least 3 independent experiments. The type strains for each capsular serovar and the strains with strong reactivities (>80%) are highlighted in dark grey. The strains presenting intermediate reactivities comprised between 30 and 60% are highlighted in light grey.

nd, not determined

609 **Table 2: Summary of capsular typing of human isolates by PCR**
610

Strain/Isolate	PCR A Primers 8244-8245	PCR B Primers 8246-8247	PCR C Primers 8274-8275	PCR D Primers 8276-8277	PCR E Primers 8278-8279	PCR ABC Primers 8296-8297	Serovar
Cc1	X		X			X	A
Cc2	X		X			X	A
Cc3	X		X			X	A
Cc5	X	X				X	A
Cc10	X		X			X	A
Cc13	X		X			X	A
Cc15	X	X				X	A
Cc21	X		X			X	A
Cc22	X		X			X	A
Cc24	X	X				X	A
Cc25	X		X			X	A
Cc6		X				X	B
Cc8		X				X	B
Cc11		X				X	B
Cc16		X				X	B
Cc17		X				X	B
Cc18		X				X	B
Cc23		X				X	B
Cc9			X			X	C
Cc14			X			X	C
Cc19			X			X	C
Cc20			X			X	C
Cc7				X			D
Cc12				X			D
Cc4					X		E

611
612 PCR positive results are represented by X.

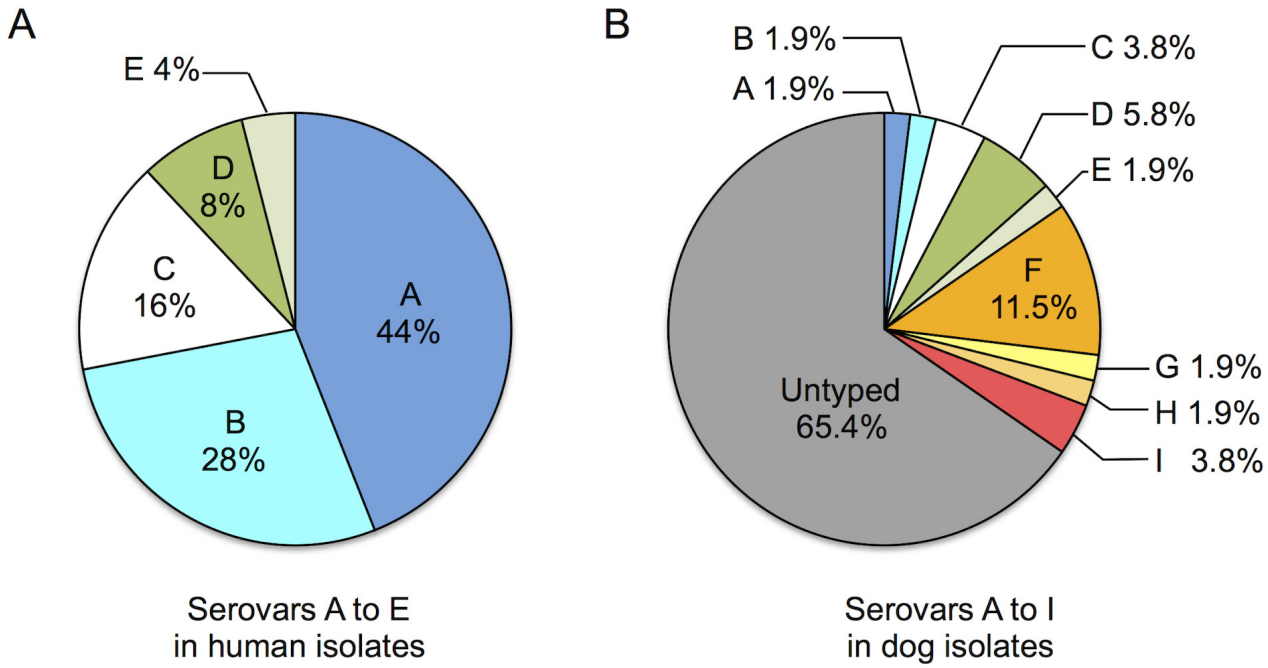
613

614 **Table 3: Interpretation of PCR typing results**
615

	PCR A Primers: 8244-8245	**PCR B** Primers: 8246-8247	**PCR C** Primers: 8274-8275	**PCR D** Primers: 8276-8277	**PCR E** Primers: 8278-8279	**PCR ABC** Primers: 8296-8297
Serovar A	X					X
	X	X				X
	X		X			X
Serovar B		X				X
Serovar C			X			X
Serovar D				X		
Serovar E					X	

616
617 PCR positive results are represented by X.

618



Serovars A to E in human isolates

Serovars A to I in dog isolates